

BIOLOGICAL NITROGEN FIXATION IN A NORTHERN TRANSVAAL SAVANNA

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ABSTRACT

The rate at which whole soil samples reduce acetylene was determined in a savanna at Nylsvley, Northern Transvaal. Activity was found from early January to mid-April. Peak activity occurred late in February. From the results it was calculated that the annual rate of nitrogen fixation probably lies between 29 and 86 kg N ha⁻¹ depending on whether, in this system, the reduction of 9 or 3 moles of acetylene are equivalent to the fixation of one mole of dinitrogen.

Nodulated legumes appear to be responsible for most if not all of the nitrogen fixation. All the legumes in the area except *Burkea africana* were found to be nodulated and capable of nitrogen fixation.

Evidence was obtained that during the acetylene reduction trials, the soil samples initially absorbed a small amount of ethylene. Although autoclaving the soil samples largely abolished the absorption, the process is considered to be a physical characteristic of the soil rather than a microbial one since it ceases after a few hours in unsterilized soil.

UITTREKSEL

BIOLOGIESE STIKSTOFBINDING IN NOORD-TRANSVAAL SAVANNA

Die tempo waarteen ongefraksioneerde grondmonsters asetileen reduseer, is in 'n savanne te Nylsvley, Noord-Transvaal bepaal. Aktiwiteit is van vroeg in Januarie tot middel April waargeneem met piekwaardes laat in Februarie. Uit die resultate blyk dit dat die jaarlikse tempo van stikstofbinding waarskynlik tussen 29 en 86 kg N ha⁻¹ is afhangende daarvan of die reduksie van 9 of 3 mol asetileen in die sisteem ekwivalent is aan die binding van een mol stikstofgas.

Genoduleerde peulplante is skynbaar vir meeste, of selfs alle stikstofbinding verantwoordelik. Daar is vasgestel dat alle peulplantsoorte in die gebied, met die uitsondering van *Burkea africana*, wortelknoppies besit en in staat is om asetileen te reduseer.

Daar is gedurende die asetileenreduksiebepalings aanduidings gevind dat die grondmonsters aanvanklik klein hoeveelhede etileen absorbeer. Alhoewel outoklaving grootliks verhoed het dat die grondmonsters daarna etileen opneem, word die etileenopname tog as 'n fisiese kenmerk van die grond eerder as 'n mikrobiologiese verskynsel gesien aangesien dit in ongestertiliseerde grond na 'n paar uur ophou.

INTRODUCTION

After Dilworth (1966) discovered that nitrogenase is capable of reducing acetylene to ethylene, Hardy *et al.* (1968) were quick to recommend that this attribute of nitrogenase be used to measure the rate of biological nitrogen fixation in the laboratory and the field. Since then Steyn and Delwiche (1970), Copley and Reuss (1972) and Vlassak *et al.* (1973) have employed the techni-

que in attempts to determine the annual rate of dinitrogen fixation in native grasslands.

The Council for Scientific and Industrial Research of South Africa is currently co-ordinating the Nylsvley Savanna Ecosystem Project (Huntley, 1978). One aspect of the project requires that an estimate be made of the rate at which nitrogen is annually being fixed in the ecosystem. This paper is a publication of the Savanna Ecosystem Project and reports on results that were obtained during 1974 to 1976 by means of the acetylene reduction method.

MATERIAL AND METHODS

Main experiment

The study was undertaken on a portion of the Nylsvley Provincial Nature Reserve in the Northern Transvaal (latitude S 24° 29', longitude E 28° 42'). The study area occurs on a plateau 1 100 m above sea level with sandy latosols and a climate that is typical of most areas occupied by savanna in the Republic of South Africa. It has a hot, wet season lasting from November through March, during which period 79 % of the mean annual precipitation of 630 mm is received (Huntley, 1978). From late April to the end of August, the climate is cool and dry while September and October are generally hot and dry.

The study area lies within Veld Type 18 of Acocks (1975) which is referred to as "Mixed Bushveld". The vegetation is an open savanna with *Burkea africana* the dominant tree species and *Eragrostis pallens* the dominant grass. Several legume species which are known to be capable of producing root nodules (Grobelaar *et al.*, 1967; Grobbelaar and Clarke, 1972, 1974, 1975) occur scattered in the grass layer in low densities.

After many preliminary studies during 1974 and 1975 the following survey procedure was finally decided on: a straight line, 399 m long was marked out in the study area by means of 400 iron pegs spaced 1 m apart. This line served as a reference line for a hypothetical rectangular grid 399 m long and 29 m wide with lines 1 m apart running parallel to the boundaries of the grid and intersecting one another at 12 000 points. Each point of intersection on the grid was a potential soil sampling point. The grid was subdivided longitudinally into 40 subsections 10 m × 29 m in size and containing 300 potential soil sampling points each. One sampling point (which had not been sampled previously) in each subsection of the grid was sampled on each sampling date, thus yielding 40 sampling points selected at random per sampling date.

For each sampling date, the sampling points on the grid were selected by means of a table of random numbers (Snedecor, 1953). In the final experiment, the grid was sampled on the following 13 occasions: 1975/10/28, 1975/11/18, 1975/12/09, 1976/01/06, 1976/01/27, 1976/02/24, 1976/03/23, 1976/04/14, 1976/05/25, 1976/06/29, 1976/08/03, 1976/09/09 and 1976/09/30.

Two cylindrical soil cores with a cross-sectional area of 25 cm² were collected about 5 cm apart at each sampling point by means of a hammer-driven stainless steel soil borer. The soil samples were taken to a depth of 40 cm (1 dm³ of soil) unless rock occurred at a shallower depth. Each soil sample was immediately hermetically sealed in a wide-mouthed glass container with a total capacity of about 1 100 cm³. The screw caps of the flasks were each fitted with a rubber serum bottle stopper.

Of the two soil samples from one sampling point, one was used as a control by not enriching it with acetylene. Approximately one tenth of the air in the other flask was replaced by commercial acetylene from a cylinder by means of the apparatus that is diagrammatically depicted in Figure 1, as follows: some of the air in flask A was withdrawn through the syringe needle B, by opening stopcock J until the pressure in the flask had dropped by the required amount as indicated by the mercury manometer C. (In practice H was mounted on the roof of a Volkswagen minibus while A, C, D and E were set up inside the rear luggage compartment of the minibus and F and G stood on the ground.) After it had been established that the flask did not leak, acetylene which had been scrubbed in concentrated H₂SO₄ to remove acetone vapours, was allowed to enter flask A through stopcock K until the gas pressure inside the flask became equal to atmospheric pressure.

The flask was disconnected from the syringe needle and shaken for a few seconds in order to facilitate the mixing of the acetylene, air and soil in the flask. A 2 cm³ sample of the gas phase was now withdrawn from the control flask as well as from the acetylene-enriched flask by means of a gas syringe. The gas samples were introduced into 2 cm³ glass serum bottles by the displacement of water (see Figure 2A) and sealed under water by means of a rubber serum bottle stopper.

The flasks were incubated in the shade at ambient temperature for 4 h. After the first 1.5 h, a 2 cm³ sample of the gas phase was taken from each flask and stored as described above. At the end of the incubation period, i.e. after a further 2.5 h, a third 2 cm³ sample of the gas phase of each flask was taken.

The gas samples were analysed in the laboratory for their ethylene concentration by means of gas-liquid chromatography, using a 1 cm³ gas sample for each analysis. The gas samples were withdrawn from the serum bottles by displacement with water (Figure 2B). For the GLC, a 3 m Porapak R column with an internal diameter of 1.5 mm was used at 60 °C in conjunction with a flame ionisation detector.

The volume of gas that was enclosed in the flask with the soil was calculated from the difference between the total volume of the flask and the volume of the soil sample. The latter was determined by displacing water in a measuring cylinder.

In calculating the rate of nitrogen fixation, it was assumed that for every 3 to

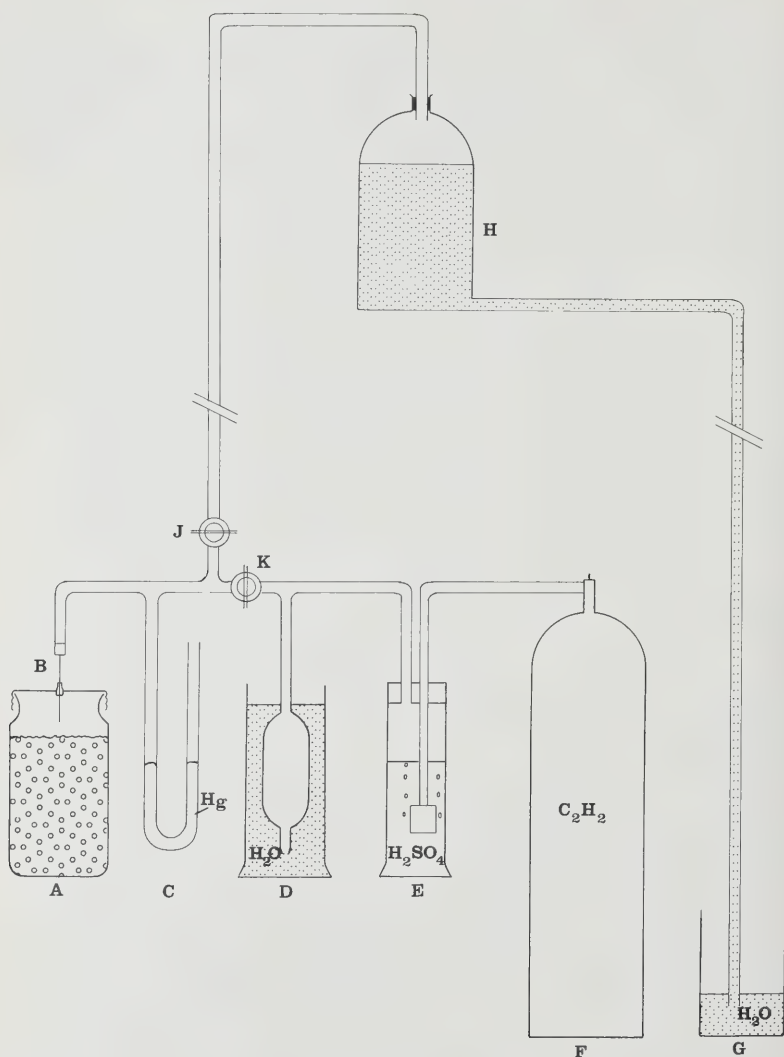


FIG. 1.

Diagrammatic representation of apparatus used in the field for replacing about one tenth of the air enclosed within a soil sample, by acetylene.

A = soil sample in flask fitted with a rubber serum stopper in its screw cap; B = syringe needle; C = mercury manometer; D = escape for excess acetylene through water; E = scrubber with conc. sulphuric acid; F = cylinder compressed acetylene; G = bucket for collecting water draining from H which is mounted about 2 m above G; J and K = stop-cocks.

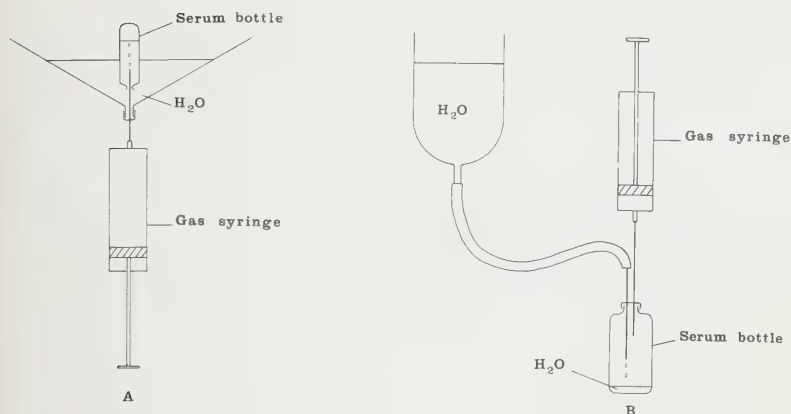


FIG. 2.

(A) Apparatus for injecting gas sample into serum bottle for storage.

(B) Withdrawal of gas sample from storage serum bottle for GC analysis.

9 moles of ethylene produced, one mole of dinitrogen would have been fixed as is claimed by Steyn and Delwiche (1970). It was also assumed that under the conditions under which the experiment was carried out, one mole of ethylene occupied 28.6 dm³.

Supplementary experiments

From preliminary experiments it became obvious that in many cases the soil samples yielded a negative rate of ethylene production—a phenomenon that could be observed due to the fact that the commercial acetylene that was used invariably contained some ethylene as an impurity. As a consequence the initial ethylene concentration varied between 4 and 18 n moles per cm³ incubation gas mixture. Changes in the rate of ethylene absorption with time were investigated. The effect of mixing the soil and acetylene containing air by briefly shaking the flask was also studied.

In another experiment, ten soil samples which absorbed ethylene were flushed with air late on the same day that the soil samples were collected in the field, by evacuating them three times by means of a water pump and allowing acetylene-free air to enter the flasks. The rubber serum bottle stoppers in the lids of the flasks were replaced by cotton wool plugs and five of the flasks were autoclaved for 1 h at 240 kPa after which all ten of the flasks were left overnight in the laboratory at room temperature. The following day, all of the flasks were re-incubated with acetylene in the usual way except that the ethylene concentration of the incubation gas mixture was increased to 300 n moles per cm³ gas mixture.

TABLE 1.

List of plant species occurring in the Nylsvley study area whose unwashed roots, shoots and soil were separately tested for acetylene reducing activity during February 1975.*

MONOCOTYLEDONES**POACEAE**

- Elionurus argenteus* Nees
- Schizachyrium sanguineum* (Retz.) Alst.
- Andropogon schirensis* Hochst.
- Cymbopogon marginatus* (Steud.) Stapf ex Burtt Davy
- Hyperthelia dissoluta* (Nees ex Steud.) Clayton
- Trachypogon spicatus* (L.f.) Kuntze
- Diheteropogon amplexans* (Nees) Clayton
- Digitaria eriantha* Steud.
- Cenchrus ciliaris* L.
- Aristida argentea* Schweick.
- A. congesta* Roem. et Schult. subsp. *congesta*
- Pennisetum patens* Gand.
- Eragrostis pallens* Hack.

COMMELINACEAE

- Commelina africana* L.

LILIACEAE

- Asparagus suaveolens* Burch.

VELLOZIACEAE

- Xerophyta retinervis* Bak.

DICOTYLEDONES**AIZOACEAE**

- Gisekia pharnaceoides* L.

ROSACEAE

- Parinari capensis* Harv.

MIMOSACEAE

- Elephantorrhiza obliqua* Burtt Davy

CAESALPINIACEAE

- Burkea africana* Hook.
- Cassia mimosoides* L.

FABACEAE

- Indigofera oxytropis* Benth.
- I. sordida* Benth.
- Tephrosia longipes* Meissn. var. *lurida* (Sond.) Gillet
- T. lupinifolia* (Burch.) DC.
- T. semiglabra* Sond.
- Rhynchosia monophylla* Schltr.

DICHAPETALACEAE

- Dichapetalum cymosum* Engl. et Prantl

TILIACEAE

- Grewia flavescens* Juss.

STERCULIACEAE

Waltheria indica L.

OCHNACEAE

Ochna pulchra Hook.

COMBRETACEAE

Terminalia sericea Burch. ex DC.

CONVOLVULACEAE

Ipomoea obscura (L.) Ker-Gawl. var. *fragilis* (Choisy) A. Meeuse

ACANTHACEAE

Crabbea hirsuta Harv.

Justicia minima A. Meeuse

RUBIACEAE

Oldenlandia herbacea (L.) Roxb.

Pygmaeothamnus zeyheri (Sond.) Robyns

* Species are arranged alphabetically within genera. The genera are arranged according to the system of de Dalla Torre and Harms (1963).

During preliminary trials, the roots of several of the common plant species were dug out and shaken free of most of the adhering sandy soil. A sample of the soil was also rapidly sieved free of roots. Samples of the shoots, roots and sieved soil were separately incubated in 300 cm³ flasks with and without acetylene as described above and tested for acetylene reduction.

RESULTS

The control soil cores without the acetylene enrichment did not release any ethylene and therefore any ethylene production was solely the result of acetylene reduction.

During the preliminary studies, it was established that all the legume species in the study area except *Burkea africana* usually have *Rhizobium*-type root nodules during the growing season and that these nodulated roots reduce acetylene at a relatively high rate (0–0.7 μ moles acetylene reduced per hour by a gram of fresh roots depending on the number and condition of the root nodules). The roots of none of the non-legume species tested (see Table 1) produced clear evidence of acetylene reducing activity. In no case did the shoots or sieved soil reduce acetylene.

Although the rate and duration of ethylene absorption varied considerably from one soil sample to the next, the results depicted in Figure 3 are typical of the majority of cases which did not show any acetylene reducing activity. By shaking the flask immediately after introducing the acetylene, the absorption of ethylene can be restricted almost wholly to the first 1.5 h of the incubation period during which time the nitrogen-fixing activity of nodulated roots in the soil should not have declined significantly in most cases.

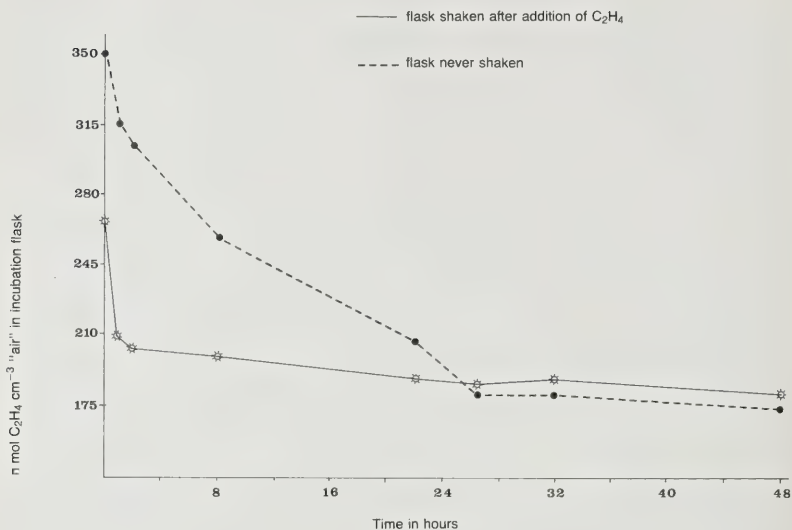


FIG. 3.

Decrease in ethylene conc. of incubation gas mixture while testing soil sample for acetylene reducing activity. In the one case (—), the flask was shaken gently for a few seconds after the ethylene was added but before the first ethylene determination was carried out. In the other case (---), the flask was never shaken. Both incubation flasks initially contained the same ethylene concentration.

From the results in Table 2, it is clear that the absorptive capacity of a soil sample can be largely restored by aerating the soil after an initial exposure to ethylene. Autoclaving the soil severely reduces the ability of the soil to absorb ethylene.

The results of the main experiment are summarised in Table 3 and the results in Table 3 which deals with the second part of the incubation periods are graphically represented in Figure 4. The acetylene reducing activity is limited mainly to the months January to April and appears to reach a peak late in February.

From Table 4 it is clear that despite an initial 1.5 h incubation period 209 of the 520 soil samples continued to show a net absorption of ethylene, albeit small amounts, during the second stage of the incubation period. Only 58 of the 520 soil samples yielded ethylene production rates in excess of 0.35μ mole per hour during the second stage of the incubation period.

TABLE 2.

Effect of ventilating and autoclaving ethylene-absorbing Nylsvey soil samples on subsequent absorption rates

Soil Sample	Rate of ethylene absorption (n mol. h ⁻¹ per soil sample*) during			
	Initial incubation		Subsequent incubation after soil ventilated	
	1st part of incub. period (1,5 h)	2nd part of incub. period (2,5 h)	1st part of incub. period (1,5 h)	2nd part of incub. period (2,5 h)
	Soil not autoclaved		Soil not autoclaved	
1	1 713	49	734	119
2	804	10	664	182
3	804	7	839	91
4	734	24	699	101
5	629	7	874	66
Mean:	937	19	762	112
	Soil not autoclaved		Soil autoclaved	
6	1 783	45	280	-164
7	1 748	- 7	245	- 10
8	1 189	-10	315	-133
9	1 049	10	-559	133
10	734	66	245	- 17
Mean:	1 301	21	105	-38

* Cylindrical soil sample with 25 cm² cross sectional area 40 cm long.

Sampling sites that yielded strong positive results initially could not be correlated with the presence of any particular plant species or other feature. In fact, at only 4 of the 58 sampling points referred to above, was a nodulating legume species recorded within a metre of the sampling point.

On one occasion those soil samples that yielded strongly positive results were carefully searched for legume root nodules. In seven of the ten cases legume-like root nodules were found despite the fact that legumes were not observed within a metre of the sampling points. A day after the initial soil samples were taken, three soil samples were collected from the immediate vicinity of 6 "active sites" and 6 "inactive sites". Some of the new soil samples were active and others inactive, irrespective of whether they were taken in the vicinity of a sampling point which initially provided an active or an inactive soil core.

On several occasions active soil samples were re-incubated 24 h after they were initially collected. In all cases it was found that the acetylene reducing activity had completely disappeared. In other cases "active" soil samples were continuously incubated for 24 hours and tested periodically for acetylene reduc-

TABLE 3.

The rate of acetylene reduction by Nylsvley soil samples during different times of the year.

Sampling Date	Rate of acetylene reduction (n mol. h ⁻¹ per soil sample)*	
	First part of Incubation period (1,5 h)	Second part of incubation period (2,5 h)
1975-10-28	-1 643	0
1975-11-18	- 629	- 35
1975-12-09	- 105	0
1976-01-06	175	385
1976-01-27	594	594
1976-02-24	-35	1 469
1976-03-23	-3 357	350
1976-04-14	-1 678	839
1976-05-25	-1 923	0
1976-06-29	-2 133	35
1976-08-03	-2 063	105
1976-09-09	- 524	- 70
1976-09-30	-1 049	-210

* Each figure is the mean of 40 determinations. Cylindrical soil samples with a cross sectional area of 25 cm² and a length of 40 cm were used.

ing activity. The activity invariably decreased with time and disappeared completely within 24 h.

If the soil underneath 25 cm² surface area reduces acetylene at a constant rate of 1,5 μ moles h⁻¹, and if the reduction of 3 moles of acetylene is equivalent to the reduction of one mole of dinitrogen, this rate of acetylene reduction would amount to an annual rate of dinitrogen fixation of 490 kg ha⁻¹. In Figure 4, the area beneath the curve represents 0,176 of the area underneath the line for a constant rate of acetylene reduction of 1,5 μ mole h⁻¹ per soil sample.

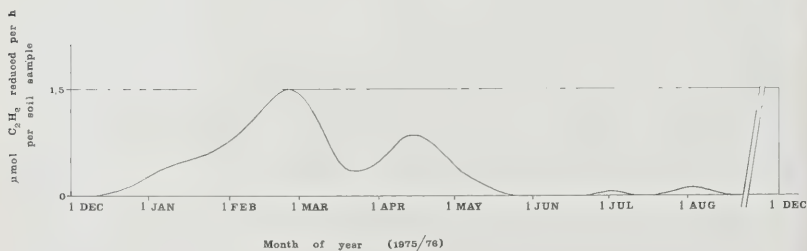


FIG. 4.

Changes in the mean acetylene reducing activity (mean of 40 determinations) of soil samples taken from Nylsvley at different times of the year. Each cylindrical soil sample had a cross sectional area of 25 cm² and a length of 40 cm.

Table 4.

Frequency distribution of acetylene reduction values obtained for soil samples at Nyilsvey.

Sampling date	Number of soil samples that yielded acetylene reduction values ($\mu\text{ mol. h}^{-1}$ per soil sample) between											
	-10,6 to -14,0	-7,1 to -10,5	-3,6 to -7,0	-0,36 to -3,5	-0,01 to -0,35	0,00 to 0,35	0,36 to 3,5	3,6 to 7,0	7,1 to 10,5	10,6 to 14,0	14,1 to 17,5	17,6 to 21,00
First part of incubation period (1,5 h)												
1975-10-28	0		1	30	8	1		0	0	0	0	0
1975-11-18	0		1	28	7	4		0	0	0	0	0
1975-12-09	0		0	1	31	8		0	0	0	0	0
1976-01-06	0		0	4	28	4	2	1	1	0	0	0
1976-01-27	0		0	3	12	15	8	0	2	0	0	0
1976-02-24	0		4	20	3	3	5	3	1	1	0	0
1976-03-23	1	5	10	14	8	1	1	0	0	0	0	0
1976-04-14	0		11	20	4	2	2	0	0	0	0	1
1976-05-25	0		3	35	1	1	0	0	0	0	0	0
1976-06-29	0		2	38	0	0	0	0	0	0	0	0
1976-08-03	0		4	34	2	0	0	0	0	0	0	0
1976-09-09	0		0	27	10	3	0	0	0	0	0	0
1976-09-30	0		0	33	6	1		0	0	0	0	0
Total	1	5	36	287	120	43	18	4	4	1	0	1
Second part of incubation period (2,5 h)												
1975-10-28	0	0	0	0	4	36	0	0	0	0	0	0
1975-11-18	0	0	0	0	29	11	0	0	0	0	0	0
1975-12-09	0	0	0	0	20	20	0	0	0	0	0	0
1976-01-06	0	0	0	0	15	19	5	1	0	0	0	0
1976-01-27	0	0	0	0	13	17	8	2	0	0	0	0
1976-02-24	0	0	0	1	6	15	13	3	1	1	0	0
1976-03-23	0	0	0		11	21	7	1	0	0	0	0
1976-04-14	0	0	0	1	10	18	10	0	0	0	0	1
1976-05-25	0	0	0	2	21	14	3	0	0	0	0	0
1976-06-29	0	0	0		16	22	2	0	0	0	0	0
1976-08-03	0	0	0		5	35	0	0	0	0	0	0
1976-09-09	0	0	0	1	23	16	0	0	0	0	0	0
1976-09-30	0	0	0	9	22	9	0	0	0	0	0	0
Total	0	0	0	14	195	253	48	7	1	1	0	1

* Cylindrical soil sample with a 25 cm² cross sectional area and a length of 40 cm.

From this, it would appear that the annual rate of dinitrogen fixation for the study site on Nylsvley is equal to about $(0,176 \times 490) = 86$ kg N per hectare. If, on the other hand as many as 9 moles of acetylene are reduced for one mole of dinitrogen fixed, the annual rate of nitrogen fixation at the Nylsvley study site approximates 29 kg N per hectare.

DISCUSSION AND CONCLUSIONS

Because the rate of ethylene absorption by soil samples declined rapidly with time, the process appears to be a physical rather than a microbial attribute of the soil despite the fact that autoclaving the soil largely abolished this characteristic.

Because of the low frequency of "active" soil samples, and the inability to predict which soil cores would yield positive results in the acetylene reduction assay, it was impractical to attempt to experimentally determine the equivalence between an acetylene reduction rate and the rate of dinitrogen fixation for the system under consideration. The widest recorded limits for such equivalence values appears to be 3 and 9 (Steyn and Delwiche, 1970). Even when an equivalence value of 9 is used, a rate of dinitrogen fixation is obtained for Nylsvley which is much higher than the annual rate of 0.4–3 kg N per hectare which other workers (Steyn and Delwiche, 1970; Copley and Reuss, 1972; Vlassak *et al.*, 1973) have recorded by means of the acetylene reduction method for North American temperate grasslands.

The relatively harsh and aerobic treatment to which the soil samples were subjected in the present study probably precluded the detection of nitrogen fixation by the type of system which Dobereiner (1973) claims to be important in tropical grasslands. Since all the available evidence points to nodulated legumes as the major, if not exclusive, dinitrogen fixing agents at the study site, the acetylene reduction method used was adapted to register its activity. In Dobereiner's work it is essential that soil cores be disturbed as little as possible or that they are pre-incubated for many hours under low oxygen tensions before the acetylene reduction assay is performed on them. Under the latter conditions the acetylene reducing activity of nodule bearing detached legume roots will be wholly lost. As it is, the present results are based on an incubation period which started 1.5 h after the soil sample was taken and in the case of some of the relatively active soil samples it was apparent that the activity started to decline during the second stage of the incubation period.

The observed presence in most of the active soil samples of legume root nodules directly supports the contention that these organs are important nitrogen fixing agents in the study site. The presence of root nodules at long distances from legume shoots is in agreement with the fact that the root systems of many of the plants in this area spread laterally for a considerable distance. In fact, it was observed that several legume species, such as *Elephantorrhiza obli-*

qua and *Cassia mimosoides* produce long rhizomes which interconnect widely spaced shoots. The decline of the acetylene reducing rate of active soil samples to virtually nil in the course of 24 h is also in agreement with the contention that legume root nodules rather than free-living nitrogen fixing bacteria are responsible for most, if not all the observed activity.

During the test period, the overall oxygen concentration in the incubation flasks could have decreased very slightly whilst the overall carbon dioxide concentration could have increased by as much as five fold (Bezuidenhout, J. J.; Department of Microbiology and Plant Pathology, University of Pretoria, pers. comm.). Although such changes should be detrimental to the activity of legume root nodules, it should stimulate the activity of certain free living nitrogen fixing bacteria and the type of system which Dobereiner (1973) has recently studied intensively. In the present case, however, the acetylene reducing activity of all the active soil samples that were incubated continuously for 24 h declined to virtually nil. It must therefore be assumed that the rates of acetylene reduction that were observed were probably wholly due to nodulated legume roots and represent an underestimation of the real rates. On the other hand, it must be borne in mind that the soil samples were generally collected late in the morning. If the acetylene reducing activity of the plants in question has a pronounced diurnal rhythm with peak activity late in the morning (as has been recorded for soybeans by Sloger *et al.* (1975)), the observed rates of acetylene reduction should be decreased by about half when it is used as the mean rate for the diurnal cycle.

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